Exhibit B

JOURNAL OF VIROLOGY, June 2001, p. 5108–5118
0022-538X/01/$04.00+0 DOI: 10.1128/JVI.75.11.5108–5118.2001
Copyright © 2001, American Society for Microbiology. All Rights Reserved.
Vol. 75, No. 11

Transfer of Hepatitis B Virus Genome by Adenovirus Vectors into Cultured Cells and Mice: Crossing the Species Barrier

MARTIN FRANZ SPRINZL, HEIKE OBERWINKLER,† HEINZ SCHALLER, AND ULRIKE PROTZER*

Zentrum für Molekulare Biologie Heidelberg (ZMBH), University of Heidelberg, D-69120 Heidelberg, Federal Republic of Germany

Received 10 November 2000/Accepted 1 March 2001

For the study of hepatitis B virus infection, no permissive cell line or small animal is available. Stably transfected cell lines and transgenic mice which contain hepadnavirus genomes produce virus, but—unlike in natural infection—from an integrated viral transcription template. To transfer hepadnavirus genomes across the species barrier, we developed adenovirus vectors in which 1.3-fold-overlength human and duck hepatitis B virus genomes were inserted. The adenovirus-mediated genome transfer efficiently initiated hepadnavirus replication from an extrachromosomal template in established cell lines, in primary hepatocytes from various species, and in the livers of mice. Following the transfer, hepatitis B virus proteins, genomic RNA, and all replicative DNA intermediates were detected. Detection of covalently closed circular DNA in hepatoma cell lines and in primary hepatocytes indicated that an intracellular replication cycle independent from the transferred linear viral genome was established. High-titer hepatitis B virions were released into the culture medium of hepatoma cells and the various primary hepatocytes. In addition, infectious virions were secreted into the sera of mice. In conclusion, adenovirus-mediated genome transfer initiated efficient hepatitis B virus replication in cultured liver cells and in the experimental animals from an extrachromosomal template. This will allow development of small-animal systems of hepatitis B virus infection and will facilitate study of pathogenicity of wild-type and mutant viruses as well as of virus-host interaction and new therapeutic approaches.

Chronic hepatitis B is one of the most common and severe viral infections of humans worldwide. Currently, 5% of the world's population are persistently infected with hepatitis B virus (HBV) (57). Infected individuals are at high risk of developing liver cirrhosis and, eventually, hepatocellular carcinoma. While an effective vaccine is available, present treatment regimens for hepatitis B are costly and often have limiting side effects (25, 60). Only about one-third of patients treated with alpha interferon show a sustained response (28, 36, 60). Nucleoside analogues do not eliminate the virus completely and may select resistant viral variants (59). The development of new treatment strategies remains a major goal but is hindered by the lack of cell lines or a small-animal model infectible with hepatitis B virus that would allow testing.

The causative agent of the disease is HBV, the prototype member of the family *Hepadnaviridae*. These small, DNA-containing viruses replicate through reverse transcription but, in contrast to retroviruses, do not integrate into the host cell genome for replication (49). Infectious virions have a lipoprotein envelope with large (L), medium (M), and small (S) envelope proteins and contain a nucleocapsid. This harbors a small (3 to 3.2 kb), partially double-stranded, relaxed circular DNA (rcDNA) genome with the viral replication enzyme, P protein, covalently attached. After entry into the host cell, the genome is delivered to the nucleus and transformed into covalently closed circular DNA (cccDNA), which serves as a template for transcription. All genomic and subgenomic transcripts are translated into protein. The mRNA for the core and the P protein serves, in addition, as an RNA pregenome. It is copackaged with P protein into newly forming capsids where it is reverse transcribed by the enzyme into DNA (for review, see references 15 and 35).

One characteristic property of the hepadnaviruses is their high species and tissue specificity: HBV infects only humans and humanoid primates or cultured primary hepatocytes of these hosts. Besides virus uptake, viral promoters and enhancers confer hepatocyte specificity during replication (15, 32). In the absence of suitable in vitro or in vivo infection systems for HBV, different experimental systems are in use to study HBV infection. Two related animal viruses are used in their natural hosts: the duck hepatitis B virus (DHBV) (46) and the woodchuck hepatitis B virus (WHV) (43). However, avian and mammalian hepadnaviruses differ in genome structure (46). Even between the closely related mammalian viruses WHV and HBV, differences, e.g., in transcriptional regulation, exist (10). Studies on immunology and pathogenesis of infection are limited by the fact that the natural hosts of these viruses, Peking ducks and woodchucks, are genetically not well defined.

Stable cell lines with integrated HBV genomes, e.g., HepG 2.2.15 cells (47), are commonly used for assessing the action of drugs on HBV replication. HBV-transgenic mice proved to be very useful for immunological studies (17). However, stable cell lines as well as transgenic mice have the disadvantage that, unlike in natural infection, HBV replicates from an integrated genome which cannot be eliminated. In addition, the level of

* Corresponding author. Present address: Hygiene Institut, Abteilung Virologie, Im Neuenheimer Feld 324, D-69120 Heidelberg, Federal Republic of Germany. Phone: 49-69221-565015. Fax: 49-69221-565003. E-mail: u.protzer@zmbh.uni-heidelberg.de.

† Present address: Department of Virology, Hygiene Institute, University of Heidelberg, D-69120 Heidelberg, Federal Republic of Germany.

virus replication cannot be varied, e.g., by varying the number of infected cells or the number of transcription templates per cell. Direct injection of naked DNA into the livers of mice or rats (11, 12, 53), in which the virus cannot spread, reaches only a minor part of the hepatocytes. A tree shrew species, *Tupaia belangeri*, was reported to be susceptible to HBV infection (55, 58) but only transiently carries the virus. Recent developments include immunodeficient mice in which stably transfected HBV-producing immortalized liver cells (5) or human hepatocytes which can be infected with HBV (38) are engrafted or whose livers are repopulated with xenogeneic hepatocytes (40). These models are promising but very laborious. Thus, there still is an urgent need for the development of cell culture and experimental animal systems which will help us to understand virus-host interaction of HBV infection, to determine pathogenicity of mutant viruses, and to test new antivirals and alternative therapeutic approaches for chronic infection.

We report here the use of adenovirus vectors to transfer replication-competent hepatitis B virus genomes into liver cells and to initiate hepatitis B virus replication in cell culture and in experimental animals. Adenovirus vectors transfer DNA efficiently and dose dependently into a broad range of resting or dividing cells of various species (27, 42). We generated adenovirus vectors in which the E1 region is replaced by replication-competent hepatitis B virus genomes and a reporter gene. DHBV and HBV replication was initiated in primary hepatocytes and hepatoma cells across the species barrier when genomes were transduced via adenovirus vectors. Furthermore, following the adenovirus-mediated genome transfer, infectious DHBV and HBV virions were released from hepatoma cells and from primary hepatocytes of different species, including mice and rats, and were secreted in vivo into the bloodstream of mice.

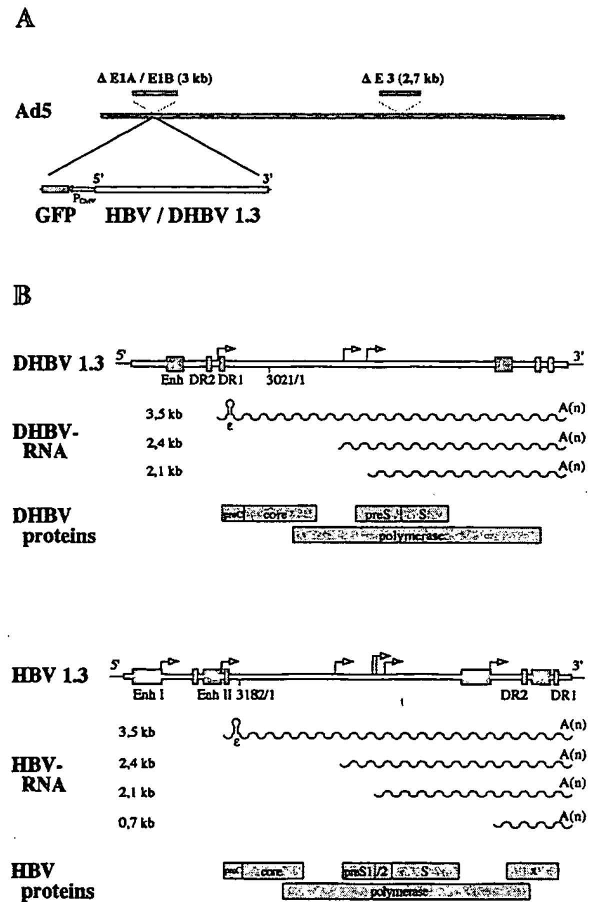

FIG. 1. Adenovirus vectors for the transfer HBV and DHBV genomes. (A) Schematic representation of the recombinant adenovirus genomes used to generate adenovirus vectors. Replication-competent HBV or DHBV genomes were inserted into the E1 region of an adenovirus subtype 5 genome (with deletions of E1A/E1B and E3) upstream of a cytomegalovirus promoter-driven GFP marker gene as described recently (20). (B) HBV- and DHBV-derived constructs comprising 1.3-fold-overlength genomes. As depicted schematically, the viral genomes have a 5′ terminal redundancy containing enhancer elements (Enh) and direct repeats DR1 and DR2, as well as the promoter and transcription initiation site of the pregenomic RNA. From these constructs, the pregenomic (3.5 kb) RNA containing the encapsidation signal ε as well as the subgenomic RNAs (2.4 and 2.1 kb) is transcribed. Thus, all viral proteins are expressed under the control of the endogenous hepadnavirus promoter/enhancer elements. A(n), polyadenylation.

MATERIALS AND METHODS

Plasmid constructs. The parental plasmid for HBV constructs was pHBV1.3 containing a 1.3-fold-overlength genome of HBV, subtype *ayw*, with a 5′ terminal redundancy encompassing enhancers I and II, the origin of replication (direct repeats DR1 and DR2), the X- and pregenomic/core promoter regions, the transcription initiation site of the pregenomic RNA, the unique polyadenylation site, and the entire X open reading frame as depicted in Fig. 1B. This construct has been proven to initiate replication of HBV efficiently and with high liver specificity in transfection experiments and in transgenic mice (18). In pHBV1.3L$^-$, artifical stop codons at the 5′ ends of the L and M protein open reading frames were introduced by exchanging nucleotides 1003 and 1279 (numbering from the core initiation codon with the A residue equal to 1) (39). The parental plasmid for DHBV constructs was pDHBV1.3, containing an analogous 1.3-fold-overlength genome of DHBV 16 (34) in which the 5′ terminal redundancy encompasses the enhancer, direct repeats DR1 and DR2, the transcription initiation site of the pregenomic RNA, and the polyadenylation site (Fig. 1B). Upon transfection into chicken hepatoma LMH cells (7), this construct leads to efficient production of infectious DHBV (33). In construct pDHBV1.3L$^-$, nucleotides 823 and 1165 were exchanged to introduce stop codons in the in pre-S region of the open reading frames of L and S proteins.

Generation of adenovirus vectors for the transfer of hepadnavirus genomes. For the generation of recombinant adenovirus genomes, the AdEasy system (20), which allows production of homogeneous adenoviruses without plaque purification, was employed. Transduction efficiency of cells can easily be followed by green fluorescent protein (GFP) expression (20). All adenovirus plasmid constructs were generously provided by Tong-Chuan He and Bert Vogelstein, Howard Hughes Medical Institute, Baltimore, Md. Then 1.3-fold-overlength DHBV and HBV genomes were excised via flanking restriction sites and cloned into the multiple-cloning site of adenovirus shuttle plasmid pAdTrack (Fig. 1A). Recombinant adenovirus genomes were obtained by homologous recombination of the shuttle plasmids and adenovirus backbone plasmid pAdEasy1, which contained an incomplete Ad5 genome with a deletion of E1 and E3 in *Escherichia coli* BJ 5183. Linearized recombinant adenovirus genomes were transfected into 293 cells, allowing propagation of the recombinant adenoviruses by *trans*-complementation of lacking E1 gene products (20). Transfection efficiency and spread of newly generated recombinant adenoviruses were followed by GFP expression monitored by fluorescence microscopy. The recombinant adenoviruses obtained were designated AdDHBV, AdDHBV-L$^-$, AdHBV, and AdHBV-L$^-$.

Preparation of recombinant adenovirus stocks. Infectious titers of the recombinant adenoviruses released into the cell culture medium or obtained from cell lysates were measured by infecting 293 cells with a dilution series of the respective virus stock. The relative number of GFP-expressing cells after 16 to 24 h determined the titer of infectious GFP-expressing viruses designated as expression-forming units (efu). In addition, the cells were monitored for a cytopathic

effect after 36 to 48 h. Recombinant adenovirus stocks used in the cell culture experiments described were obtained by synchronous infection of 293 cells at 80% confluency with a multiplicity of infection (MOI) of 5 efu per cell. When >90% of the cells showed a cytopathic effect (usually after 48 to 72 h), cells were harvested in their culture medium and subjected to three freeze/thaw cycles. After sedimentation of the cell debris, supernatant containing the adenovirus particles was used as the recombinant adenovirus stock and stored in small aliquots at −70°C after addition of 10% glycerol. Repeated freezing and thawing were avoided. Titers of the stocks obtained were between 2.5×10^8 and 2.5×10^9 efu/ml. Recombinant AdDHBV stocks used for the infection of mice were obtained from 7×10^7 and 1.5×10^8 293 cells infected as described above. When all cells showed a cytopathic effect, they were collected by centrifugation at $250 \times g$ and stored in 10 mM Tris, pH 8.0, at −70°C. Recombinant adenoviruses were released by three cycles of rapid freezing and thawing and, after removal of the cell debris, purified by sedimentation through a cesium chloride gradient (L8-M Ultracentrifuge; Beckman Instruments, Munich, Federal Republic of Germany) (SW40 Ti rotor, 32,000 U/min, 20°C, 20 h). Purified virus was dialyzed extensively against virus storage buffer (137 mM NaCl, 5 mM KCl, 10 mM Tris [pH 7.4], 1 mM $MgCl_2$) and stored in small aliquots after addition of 10% glycerol at −70°C until further use. Titers of the purified recombinant adenoviruses obtained were usually between 2.5×10^{10} and 1×10^{11} efu/ml.

Cells and cell culture conditions used. 293 cells were maintained in minimum essential Eagle medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 50 IU of penicillin/ml, and 50 μg of streptomycin/ml at 37°C in 5% CO_2. Chicken hepatoma LMH cells were maintained in Dulbecco's modified Eagle's medium-nutrient mixture Ham F-12 medium supplemented with 10% FCS, 2 mM L-glutamine, 50 IU of penicillin/ml, 50 μg of streptomycin/ml, 1 mM sodium pyruvate, and nonessential amino acids (Gibco BRL Life Technologies, Karlsruhe, Federal Republic of Germany). Human hepatoma HepG2 cells (52) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 2 mM L-glutamine, 50 IU of penicillin/ml, 50 μg of streptomycin/ml, 1 mM sodium pyruvate, and nonessential amino acids (Gibco BRL Life Technologies). Primary hepatocytes were isolated by a standard two-step collagenase perfusion and subsequent differential centrifugation essentially as described previously (14, 41). Surgical human liver biopsies were perfused via a large branch of the portal vein after disclosure of smaller vessels. Livers from 2- to 3-week-old Peking ducks, 16- to 20-week-old C57BL/6 mice, 2- to 4-month-old Sprague-Dawley rats, and 6- to 12-month-old *Tupaia belangeri* tree shrews were perfused via the portal vein. When a homogenous liver cell suspension had been obtained, hepatocytes were sedimented three times at $50 \times g$ before plating onto 6- or 12-well plates or 100-mm-diameter plastic dishes. Primary human, rat, and *Tupaia* hepatocytes were seeded at a density of 1.75×10^5 to 2×10^5 cells/cm^2, and primary mouse hepatocytes were seeded at a density of 0.75×10^5 to 1×10^5 cells/cm^2 onto collagen type I (Sigma Aldrich, Irvine, Calif.)-coated tissue culture plates in supplemented Williams E medium (50 μg of gentamicin/ml, 50 μg of streptomycin/ml, 50 IU of penicillin/ml, 2.25 mM L-glutamine, 0.06% glucose, 23 mM HEPES [pH 7.4], 4.8 μg of hydrocortisone/ml, 1 μg of inosine/ml, 1.5% dimethyl sulfoxide) with 10% FCS. Primary duck hepatocytes (2.5×10^5 cells/cm^2) were seeded without FCS on untreated cell culture dishes. Primary cells were maintained at 37°C and 5% CO_2 in the supplemented Williams E medium without addition of FCS. Surgical human liver biopsies were obtained with informed consent of the donor as approved by the local ethics committee. Animal experiments were approved by the local authority, and all animals received human care in accordance with the National Institutes of Health guidelines.

Infection of cells and animals. Cells were infected with the proper recombinant adenovirus at 80% confluency at day 1 or 2 postseeding. The amount of virus was chosen to achieve GFP expression after 24 h in approximately 90% of the cells. This allowed us to transduce the cells with a constant, low amount of the hepadnavirus genomes. Thus, primary mouse and duck hepatocytes were usually infected with an MOI of 50 efu/cell; HepG2 cells and rat and *Tupaia* hepatocytes with an MOI of 20 efu/cell; and primary human hepatocytes with an MOI of 3 efu/cell. In general, the MOI had to be optimized for each cell preparation. Eight- to 12-week-old male C57BL/6 mice were infected by a single injection of 2×10^9 efu of purified AdDHBV into the tail vein. Mice were sacrificed at day 5 after inoculation and were bled to prepare serum, and their livers were snap frozen in liquid nitrogen and stored at −70°C for subsequent analyses (see below). For infection with HBV or DHBV, primary human or duck hepatocytes, respectively, were incubated for 24 h with virus stocks diluted at an appropriate MOI in supplemented Williams E medium. If not otherwise indicated, for infection of duck hepatoctyes and of human hepatocytes, respective MOIs of 20 DHBV DNA genome equivalents and of 100 HBV DNA genome equivalents were used. All infections were stopped by thorough washing with Hanks' buffered salt solution.

Assays detecting hepadnavirus infection. (i) Analysis of secreted HBV antigens. HBsAg and hepatitis B e antigen (HBeAg) were determined by commercially available assays (Abbott Laboratories, Abbott Park, Ill.) in the cell culture medium after removal of cell debris and storage at −20°C until analysis.

(ii) Immunofluorescence staining of intracellular hepadnavirus proteins. Cell monolayers were fixed with 100% methanol at −14°C at day 4 postinfection (p.i.). The number of hepadnavirus-infected or hepadnavirus-replicating cells was determined by immunofluorescence staining of intracellular hepadnavirus antigens. For detection of DHBV and of HBV, polyclonal rabbit antisera recognizing both the DHBV core protein (45) and the pre-S domain of DHBV L protein (44) and a polyclonal antiserum recognizing HBV core protein (2) were used, respectively. As a secondary antibody, Alexa 488- or Alexa 568-labeled goat anti-rabbit immunoglobulins G were used (Dianova, Hamburg, Federal Republic of Germany).

(iii) Western blot analysis of intracellular hepadnavirus proteins. For Western blot analysis of intracellular HBV and DHBV protein, 10^6 cells were lysed at day 4 or 6 p.i. in 250 μl of protein-sample buffer (200 mM Tris-Cl [pH 8.8], 10% glucose, 5 mM EDTA, 0.1% bromphenol blue, 3% sodium dodecyl sulfate [SDS], 2% β-mercaptoethanol). Fifty milligrams of frozen mouse liver tissue was pulverized using a microdismembrator and lysed in 1 ml of protein-sample buffer. Twenty-five microliters of the respective lysate (equivalent to 10^5 cells or 2.5 μg of liver tissue) was separated by SDS–12.5% (for HBV proteins) or –10% (for DHBV proteins) polyacrylamide gel electrophoresis and blotted onto a polyvinylidene difluoride membrane. Hepadnavirus proteins were detected using polyclonal rabbit antisera against DHBV core protein (45) or against HBV core protein (2), monoclonal mouse antibody 4F8 recognizing amino acids 100 to 105 of the pre-S domain of DHBV L protein (kindly provided by Christa Kuhn, University of Heidelberg, Heidelberg, Federal Republic of Germany) or MA18/7 (21) recognizing p39 and gp42 of HBV L protein (kindly provided by Klaus-Hinrich Heermann, University of Göttingen, Göttingen, Federal Republic of Germany) and the respective peroxidase-conjugated secondary antibody (Dianova). Protein bands were visualized using the enhanced chemiluminescence or the enhanced chemifluorescence system (Amersham, Cleveland, Ohio).

(iv) Southern blot analysis of intracellular viral DNA. Cells from a 100-mm-diameter cell culture dish were lysed in 50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 150 mM NaCl, and 0.1% SDS, and total DNA was extracted after proteinase K digestion. Non-protein-bound low-molecular-weight DNA, which includes hepadnavirus cccDNA, was isolated by a modification of the Hirt lysis method as described (50). Briefly, cells were lysed in 2 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 150 mM NaCl, 1% SDS). Protein-bound DNA was precipitated on ice for 5 min after addition of KCl to a final concentration of 0.5 M and was removed by centrifugation. Non-protein-bound DNA was isolated from the supernatant by phenol-chloroform extraction after proteinase K digestion, ethanol precipitated, and dissolved in 10 mM Tris–0.1 mM EDTA, pH 8.0. After RNase digestion (5 μg/ml, 15 min, 37°C), 10% of the total and 25 to 50% of the cccDNA preparation were analyzed by Southern blotting and DHBV or HBV DNA was detected by a ^{32}P-labeled DHBV or HBV DNA probe (specific activity, $\sim 10^8$ counts/μg), respectively.

(v) Northern blot analysis of intracellular viral RNA. Intracellular hepadnavirus RNA was analyzed by Northern blotting. Total RNA was prepared from a 100-mm-diameter cell culture dish, and mRNA was purified using oligo(dT)$_{25}$-coated magnetic beads (Dynabeads; Dynal, Oslo, Norway). Fifteen micrograms of the mRNA was size fractionated by electrophoresis through a 1.0% formaldehyde agarose gel and blotted onto a nylon membrane. Viral RNAs were detected using the appropriate ^{32}P-labeled DNA probe.

(vi) DNA dot blot analysis of progeny hepadnavirus DNA. Hepadnavirus particles contained in 2 or 4 ml of cell culture medium (according to 2×10^6 cells) were sedimented through a cesium chloride step gradient (density, 1.15 to 1.4 g/ml) to separate unenveloped capsids and adenovirus particles from enveloped hepadnaviruses (37). Gradient fractions were collected from the bottom. Hepadnavirus DNA was detected by dot blot hybridization with an appropriate ^{32}P-labeled DNA probe and quantified on a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.) using a dilution series of DHBV DNA or HBV DNA, respectively, as a standard.

(vii) Analysis of serum- and liver-derived DNA by PCR. Total DNA was extracted from 50 mg of pulverized frozen mouse liver as described above and from 50 μl of mouse serum using microspin columns (QIAamp Blood Kit; Qiagen GmbH, Hilden, Federal Republic of Germany). PCR was performed to detect DHBV DNA with upstream primer DHBV 1031 (5′-TCCGACTCCTCA AGAGATTC-3′) and downstream primer DHBV 1581 (5′-CGTTGTCCGTCA GATACAGCA-3′) and to detect adenovirus DNA with upstream primer Ad

TABLE 1. Transduction rates of 293 cells, HepG2 cells, and primary duck hepatocytes for AdHBV-L⁻ at various MOIs[a]

Cell type	MOI	% GFP-expressing cells (avg ± SD)
293	0.6	58.9 ± 5.5
	1.2	78.9 ± 1.3
	2.5	93.6 ± 2.7
	5	98.3 ± 1.1
HepG2	5	48.6 ± 2.2
	10	67.5 ± 1.8
	20[b]	85.9 ± 2.4
	40	91.7 ± 0.9
PDH[c]	12.5	19.8 ± 0.88
	25	37.3 ± 3.44
	50[b]	49.6 ± 2.52
	100	88.9 ± 2.15

[a] The percentage of transduced cells was determined by fluorescence-activated cell sorter analysis of GFP-expressing cells.
[b] MOI used for further experiments.
[c] PDH, primary duck hepatocytes.

4325 (5'-TCAGTAGCAAGCTGATTGCC-3') and downstream primer Ad 4751 (5'-ACCATTATACCGCAGTCTG-3'). Single-round (nonnested) PCR was carried out with 5% of the extracted DNA in a 50-μl volume containing 100 pmol of each primer, 250 μM concentrations of each nucleotide, 1.5 mM $MgCl_2$, and 1.25 U of *Taq* polymerase (MBI Fermentas, Vilnius, Lithuania) in 1× PCR buffer provided by the manufacturer. The following temperature profile was used: cycle 1, 5 min at 94°C, 1 min at 56°C, 1 min at 72°; cycles 2 to 39, 1 min at 94°C, 1 min at 56°C, 1 min at 72°C; and cycle 40, 1 min at 94°C, 1 min at 56°C, 4 min at 72°C. Ten microliters of the amplification product was analyzed by electrophoresis in an ethidium bromide-stained 1.5% agarose gel. By this protocol, 10^2 molecules of a DNA standard subjected to PCR amplification were easily detected.

RESULTS

Adenovirus vectors allow efficient and dose-dependent transfer of hepadnavirus genomes. In the adenovirus vectors (Fig. 1A), a linear, replication-competent HBV or DHBV 1.3-fold-overlength construct (Fig. 1B) together with a cytomegalovirus promoter-driven GFP gene was inserted replacing the E1 region. Titers of AdDHBV and AdHBV obtained were two- to fivefold lower than titers of recombinant adenoviruses containing only the GFP expression cassette. Table 1 exemplifies infection efficacies for 293 cells, HepG2 cells, and primary duck hepatocytes using AdHBV-L⁻. Infection rates were determined by fluorescence-activated cell sorter analysis of GFP-expressing cells in three independent experiments. GFP expression correlated with expression of hepadnavirus proteins (as determined by immunofluorescence staining) in the vast majority (>90%) of single cells (data not shown).

The amount of virus used for infection experiments was optimized for each cell preparation in order to transduce 80 to 90% of the cells in each individual experiment as determined by GFP expression. This ensured that cells were transduced with a constant copy number of the hepadnavirus genomes. In addition, cytotoxicity due to GFP expression (31) or toxic adenovirus proteins was minimized. The MOI necessary to obtain transduction rates of 80 to 90% varied considerably between the different cells used, with the rodent and avian cells (MOI, 50 efu/cell) showing the lowest and human hepatocytes (MOI, 3 efu/cell) showing the highest permissivity. Using these conditions, in less than 10% of the cells a cytopathic effect was observed (determined by crystal violet staining of living cells at day 7 posttransduction; data not shown). Light microscopy showed little or no alteration of transduced cells (see below).

DHBV replication following adenovirus DHBV genome transfer into primary duck hepatocytes. To test whether hepadnavirus constructs were fully functional in the context of the recombinant adenovirus genome, we infected cultured primary duck hepatocytes with AdDHBV and AdDHBV-L⁻, as these are permissive to DHBV infection and efficiently initiate DHBV replication (54). DHBV-L⁻ was used because it established the full intracellular replication cycle without release of infectious DHBV which could spread to neighboring cells (50).

Following adenovirus-mediated transfer of 1.3-fold-overlength DHBV genomes, we observed transcription of pregenomic and subgenomic DHBV RNAs by Northern blot analysis of total RNA after 48 h (Fig. 2A, left panel). DHBV core protein was expressed from DHBV wild-type as well as from DHBV-L⁻ genomes, whereas L protein, as expected, was expressed only from DHBV wild-type genomes (Fig. 2A, middle and right panels; Western blot analysis of cellular lysates taken at day 4 posttransduction). By Southern blot analysis of total DNA extracted from cells transduced with AdDHBV-L⁻ (Fig. 2B), all replicative DHBV DNA intermediates (rc-, single-stranded [ss-], linear, and cccDNA) were detected. As in infection experiments with serum-derived DHBV, DHBV replicative intermediates and DHBV proteins were first detected at day 2 posttransduction with AdDHBV, and DHBV particles were released into the cell culture medium from day 3 posttransduction on (data not shown).

To determine whether DHBV replication independent from the transferred, linearized DHBV genome was established in the transduced cells, we tested for the presence of DHBV cccDNA in time course experiments using AdDHBV-L⁻. This excluded the spread of progeny DHBV and allowed us to focus on DHBV replication in the transduced cells. DHBV cccDNA was detected from day 2 onwards, in increasing amounts, by Southern blot analysis (Fig. 2C). As described for infection with a mutant DHBV defective in envelope protein, cells accumulated high levels of cccDNA due to the lack of regulatory L protein (50, 51). The time course of establishment of cccDNA was identical to that observed after infection with serum-derived DHBV (data not shown). Taken together, adenovirus-mediated genome transfer established DHBV replication very similar to that observed following natural infection.

HBV replication following adenovirus HBV genome transfer into primary human hepatocytes. Cultured primary human hepatocytes are infectible with HBV and support the essential steps of HBV replication (13, 16). We used primary human hepatocytes to investigate HBV replication after transduction with AdHBV and AdHBV-L⁻. Eighty to 90% of the human hepatocytes were transduced with the adenovirus vectors at an MOI of 3 to 5 efu/cell, with little interexperimental variability (as determined by GFP expression). AdHBV-L⁻ was used as a control to exclude spread of infectious HBV to neighboring cells. Following adenovirus-mediated HBV genome transfer, pregenomic and subgenomic HBV RNAs were detected in cellular lysates in a ratio expected from natural infection (data not shown). HBV core protein (p22; Fig. 3A, left panel) was detected in lysates of cells transduced with HBV wild-type and

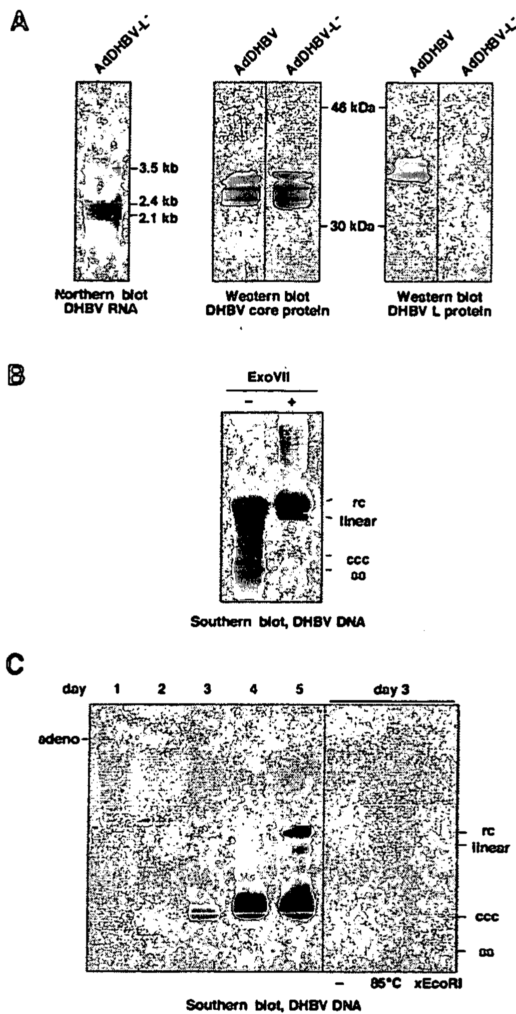

FIG. 2. DHBV replication in primary duck hepatocytes following AdDHBV genome transfer. Primary duck hepatocytes were transduced with AdDHBV or AdDHBV-L$^-$ at an MOI of 50 efu/cell. (A) Pregenomic (3.5 kb) and subgenomic (2.4 and 2.1 kb) RNAs were detected by Northern blot analysis of total RNA 48 h after infection (left panel). In AdDHBV-transduced cells, DHBV core protein (middle panel) and L protein (right panel) were detected, whereas in AdDHBV-L$^-$-transduced cells core, but no L, protein was detected (Western blot analysis of cellular lysates taken at day 4 posttransduction). Molecular masses are given in kilodaltons. (B) Southern blot analysis of total DNA extracted from AdDHBV-L$^-$-transduced cells (day 4 posttransduction) demonstrated reverse transcription of DHBV pregenomic RNA into double-stranded rcDNA (rc) and linear DNA as well as into ssDNA (ss) intermediates. Digestion of the extracted DNA with ss-specific exonuclease VII (ExoVII) revealed that DHBV cccDNA was also present. (C) Southern blot analysis of DHBV DNA extracted after a modified Hirt lysis (see Materials and Methods). DHBV cccDNA became first detectable at day 2 p.i. with AdDHBV-L$^-$ and was hyperamplified during the next days due to the lack of L protein. The right panel shows a repeated analysis of the lysate taken at day 3 p.i. (−). DHBV cccDNA is heat stable (85°C) and, like rcDNA, is linearized by *Eco*RI (xEcoRI). Bands corresponding to the expected size of rc-, linear, ccc-, and ssDNA are indicated; high-molecular-weight bands correspond to the recombinant adenovirus genome (adeno; for analysis see Fig. 5 legend).

HBV-L$^-$ genomes, whereas L protein (p39 and gp42) was only detected in lysates of cells transduced with HBV wild-type genomes (Fig. 3A, right panel).

To determine whether HBV replication would occur independent from the transferred, linearized HBV genome as observed with DHBV, we tested for the presence of HBV cccDNA. DNA extracted following a modified Hirt lysis of AdHBV and AdHBV-L$^-$-transduced primary human hepatocytes was analyzed by Southern blot analysis using a ^{32}P-labeled HBV DNA probe. Following transduction with AdHBV and AdHBV-L$^-$, HBV cccDNA was detected (Fig. 3B). As in an HBV-infected human liver analyzed in parallel, cccDNA proved to be heat stable (85°C for 5 min) in contrast to coisolated rcDNA, which is denatured to an ss form (19). This indicated that HBV replication was established, at least in part, as independent from the linear HBV genome transduced. The lack of L protein retained HBV rcDNA in the cells but did not lead to hyperamplification of HBV cccDNA, in contrast to AdDHBV infection.

Particles released into the cell culture medium of AdHBV-transduced cells were analyzed by sedimentation into a cesium chloride gradient and subsequent dot blot analysis using a ^{32}P-labeled HBV DNA probe. Enveloped HBV particles (buoyant density, 1.24 to 1.27 g/cm^3) were detected from day 3 to 4 posttransduction (Fig. 3C). Quantitation in comparison to an HBV-DNA standard applied to the same blot by using a PhosphorImager revealed that up to 150 enveloped HBV particles per cell per day were released by the human hepatocytes (data not shown). Following AdHBV-L$^-$ infection of primary human hepatocytes, unenveloped HBV nucleocapsids (buoyant density, 1.30 to 1.36 g/cm^3), but no enveloped virions, were released from the cells (Fig. 3C). The mechanism by which naked capsids are released remains unknown. The weak signals detected throughout the gradient may be caused by background hybridization as well as by unspecific association of the capsids to cellular membranes (30). When the adenovirus inoculum was removed by thorough washing, no particles sedimented at the bottom of gradient, where adenovirus particles were expected (buoyant density, 1.32 to 1.35 g/cm^3) (data not shown). Daily inspection by light microscopy proved the integrity of the majority of the infected cells. As shown in Fig. 3D, the morphology of transduced cells did not significantly change up to 2 weeks after isolation. In conclusion, AdHBV initiated efficient HBV replication in primary human hepatocytes and induced establishment of the natural transcription template, HBV cccDNA.

Kinetics of HBV replication in human hepatoma HepG2 cells after adenovirus genome transfer. To determine the kinetics of HBV replication after adenovirus-mediated HBV genome transfer, we used human hepatoma HepG2 cells. HepG2 cells have been described as efficiently supporting HBV replication and establishing a complete intracellular HBV replication cycle after transfection or baculovirus vector-mediated transfer of HBV genomes (8, 52).

Following transduction with AdHBV at an MOI of 20 efu/cell, intracellular HBV core protein was detected from day 2 onwards by Western blot analysis (Fig. 4A), while intracellular HBV L protein was detected from day 3 (data not shown). HBV replicative intermediates were detected from day 2 posttransduction in increasing amounts until day 5 posttransduc-

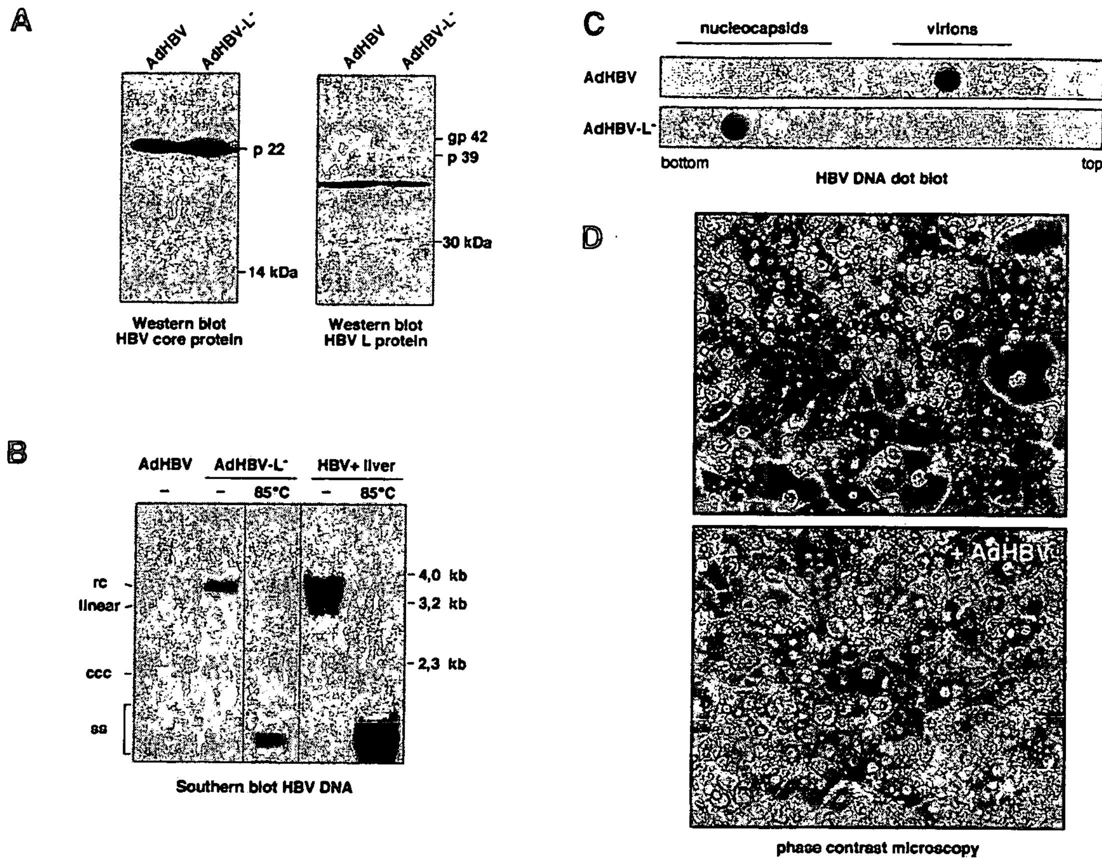

FIG. 3. HBV replication in primary human hepatocytes following AdHBV genome transfer. Primary human hepatocytes were transduced with AdHBV or AdHBV-L⁻ at an MOI of 3 efu/cell. (A) By Western blot analysis of cellular lysates taken at day 4 posttransduction, HBV core protein (p22) was detected in AdHBV- and AdHBV-L⁻-transduced cells (left panel), whereas L protein (p39 and gp42) was detected only in AdHBV-transduced cells (right panel). Molecular masses are noted at right of gels in kilodaltons. (B) Southern blot analysis of HBV DNA extracted after a modified Hirt lysis (see Materials and Methods) at day 4 posttransduction. Bands corresponding to the expected size of rc-, double-stranded linear (linear), and cccDNA, as well as the position of ssDNAs, are indicated. HBV cccDNA was detected in AdHBV- and AdHBV-L⁻-transduced cells at comparable amounts. The identity of cccDNA was proven by its heat stability (85°C) as in an HBV-infected human liver (HBV + liver). kb, kilobases. (C) The cell culture medium of AdHBV- and AdHBV-L⁻-transduced cells (collected from day 3 to 4 p.i.) was subjected to cesium chloride gradient centrifugation and subsequent HBV DNA dot blot analysis (fractions collected from bottom [δ, 1.4 g/cm^3] to top [δ, 1.15 g/cm^3]). Following AdHBV infection, HBV DNA-containing particles banded at the expected position of enveloped HBV virions. Following AdHBV-L⁻ infection, particles which banded at the expected position of unenveloped nucleocapsids were released but not any enveloped virions. (D) Light microscopy of primary human hepatocytes infected with AdHBV at an MOI of 3 efu/cell (+AdHBV) (lower panel) at day 10 posttransduction in comparison to uninfected cells cultured in parallel (upper panel).

tion by Southern blot analysis of total DNA extracted from cellular lysates (Fig. 4B). Soluble HBV antigens, HBsAg and HBeAg, first became detectable in the cell culture medium the day after transduction, increased from day 1 to day 3, and reached constant levels between day 3 and day 6 posttransduction (data not shown). Enveloped HBV particles were released into the cell culture medium from day 2 posttransduction onwards in increasing amounts, as determined by sedimentation in a cesium chloride gradient and subsequent HBV DNA dot blot analysis (Fig. 4C) (representative experiment). Progeny HBV production, as well as secretion of HBsAg and HBeAg (data not shown), reached its maximum at day 5 or 6 posttransduction and thereafter slowly decreased. The maximal amount of progeny released was determined in different experiments to vary between 30 and 100 enveloped particles per cell per day. The decrease after day 6 was paralleled by a constant loss of cells observed during long-term culture without further passaging the cells. However, a cytotoxic effect of the expressed GFP during long-term culture could not be excluded.

To test infectivity of HBV particles released by AdHBV-transduced HepG2 cells, we infected primary human hepatocytes at an MOI of 100 HBV genome equivalents per cell with HBV particles concentrated from the medium by precipitation with polyethylene glycol (41). After 4 days, about 5% of the primary human hepatocytes stained positive for HBV core protein by typical cytoplasmic immunofluorescence staining as a marker of productive HBV infection (Fig. 4D). No GFP expression was detected in the core positive cells, which would have indicated contaminating AdHBV. Infection efficacy and intracellular localization of HBV core protein were the same as in parallel infection experiments using authentic HBV produced after transfection of HuH7 cells (41) (data not shown). From this we

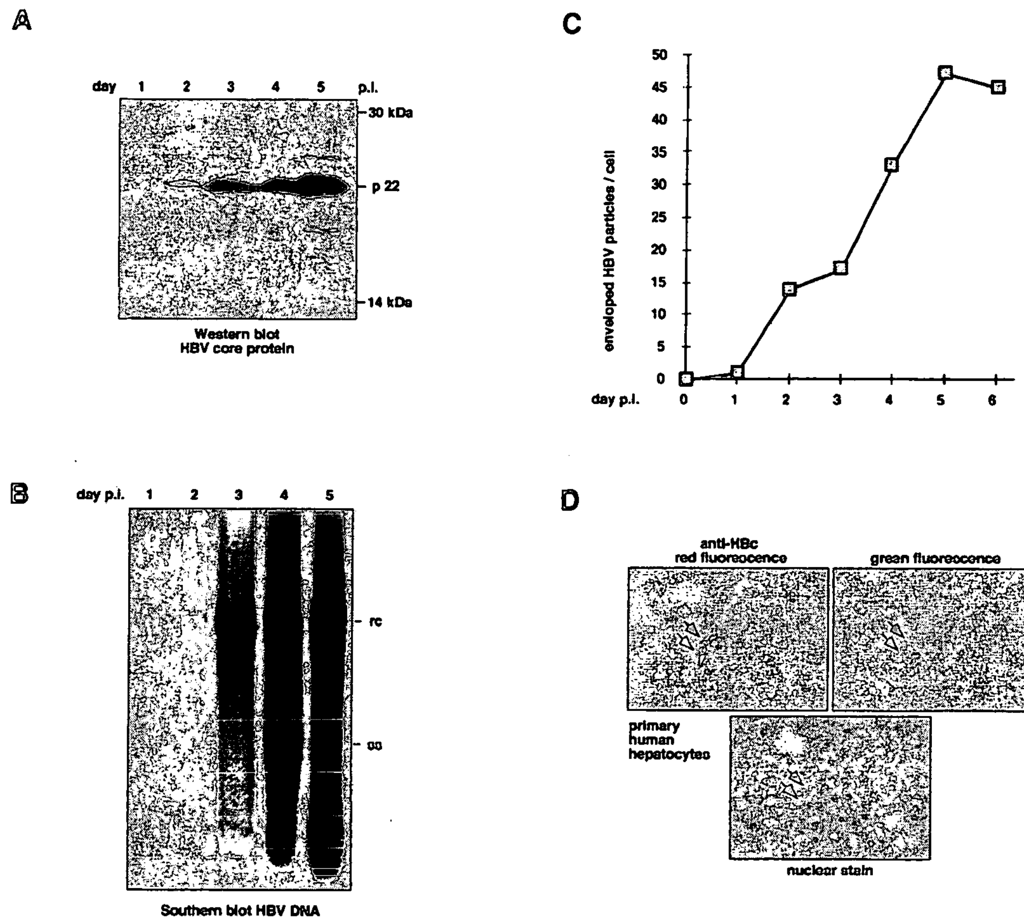

FIG. 4. Kinetics of HBV replication in human hepatoma HepG2 cells following adenovirus genome transfer. HepG2 cells were transduced with AdHBV at an MOI of 20 efu/cell at 80% confluency. (A) HBV core protein (p22) was detected from day 2 p.i. in increasing amounts by Western blot analysis of cellular lysates. Molecular sizes are given in kilodaltons at right. (B) HBV replicative DNA intermediates were detected in increasing amounts by Southern blot analysis of total DNA extracted from cells days 2 to 5 p.i. Bands corresponding to the expected size of rc- and ssDNAs are indicated. (C) Quantitative analysis of HBV released into the cell culture medium revealed that progeny virus secretion started at day 2 and steadily increased until day 5 posttransduction (HBV-DNA dot blot analysis of enveloped HBV sedimented through a cesium chloride gradient). Because the level of HBV replication varied between different experiments, a representative experiment is shown. (D) Primary human hepatocytes were infected with HBV particles released from the transduced HepG2 cells. Cells were successfully infected with HBV, as shown by immunofluorescence staining at day 4 p.i. for HBV core protein using a red fluorescent secondary antibody (upper left panel). A nuclear stain (lower panel) proved the cytoplasmic localization of HBV core protein. A lack of GFP expression (upper right panel) argued against a contamination with AdHBV, which expresses a GFP marker gene. For better illustration, immunofluorescence stains are shown in an inverse fashion.

concluded that infectious HBV was released by the AdHBV-transduced HepG2 cells.

Taken together, primary human hepatocytes as well as human hepatoma cells efficiently established HBV replication following transduction with AdHBV. Therefore, HBV gene expression and replication were, at least in part, autonomous, i.e., independent from the transferred linear genome, and led to the secretion of infectious virions.

Release of DHBV and HBV after adenovirus-mediated genome transfer into hepatocytes of various species. If HBV replication could easily and efficiently be initiated across the species barrier, for example in mouse or rat cells, this would help to solve many open questions concerning virus-host or virus-cell interaction. To test whether hepatitis B virus replication could be initiated across the species barrier by an adenovirus genome transfer, we isolated primary hepatocytes from mouse, rat, and *Tupaia*, and infected them with AdHBV and AdDHBV. In addition, primary duck hepatocytes were infected with AdHBV and primary human hepatocytes were infected with AdDHBV.

As shown in Fig. 5A, enveloped HBV particles (buoyant density, 1.24 to 1.27 g/cm^3) were released from mouse, rat, *Tupaia*, and even from duck hepatocytes following transduction with AdHBV. Accordingly, enveloped DHBV particles (buoyant density, 1.16 to 1.20 g/cm^3) were released from mouse, rat, *Tupaia*, and human hepatocytes following transduction with AdDHBV (Fig. 5B). DHBV particles released from mouse, rat, and *Tupaia* hepatocytes proved to be infectious upon passage to permissive primary duck hepatocytes, as shown by immunofluorescence staining of the infected duck

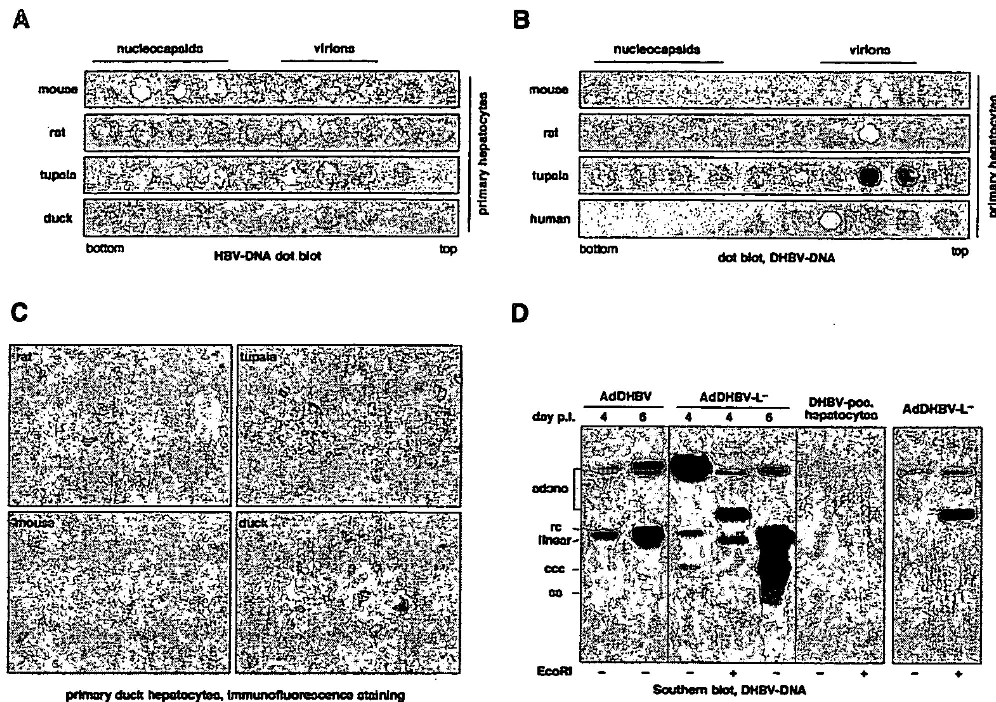

FIG. 5. Adenovirus genome transfer initiates HBV and DHBV replication in liver cells from various species. (A and B) Dot blot analysis of fractions collected from bottom (δ, 1.4 g/cm^3) to top (δ, 1.15 g/cm^3) of cesium chloride gradients of the respective cell culture medium using an HBV or DHBV DNA probe, respectively. Enveloped HBV particles (virions) were detected in the cell culture medium of AdHBV-transduced primary hepatocytes isolated from mice, rats, *Tupaia* shrews, and ducks (A). Accordingly, enveloped DHBV particles (virions) were detected in the cell culture medium of primary mouse, rat, *Tupaia*, and human hepatocytes following transduction with AdDHBV (B). (C) DHBV particles released proved infectious on primary duck hepatocytes. DHBV contained in 1 ml of medium from rat, *Tupaia*, or duck hepatocytes (collected day 3 to 4 post-AdDHBV transduction) or from mouse hepatocytes (collected day 3 to 6 post-AdDHBV transduction) was used as an inoculum. Infected duck hepatocytes were detected by immunofluorescence staining for intracellular DHBV core protein, which shows a cytoplasmic distribution (day 4 p.i.; for better illustration, immunofluorescence stains are shown in an inverse fashion). (D) Southern blot analysis of DHBV DNA in primary *Tupaia* hepatocytes. Analysis of DNA extracted at days 4 and 6 p.i. with AdDHBV or AdDHBV-L$^-$ following a modified Hirt lysis demonstrated the presence of DHBV cccDNA in primary *Tupaia* hepatocytes (DNA from 2 × 10^6 cells/lane). As a positive control, cccDNA extracted from 10^6 DHBV-positive hepatocytes isolated from an infected duckling was applied. Restriction digestion with *Eco*RI-linearized rc- and cccDNA was monitored. Bands corresponding to the expected size of rc-, linear, ccc-, and ssDNA are indicated. High-molecular-weight bands are of adenoviral origin (adeno) as shown by analysis of DNA contained in 10^6 efu of AdDHBV-L$^-$.

hepatocytes (Fig. 5C). Cells expressed no GFP, which strongly argued against the presence of AdDHBV. In addition to all other replicative DHBV intermediates, cccDNA was detected by Southern blot analysis in AdDHBV-transduced *Tupaia* hepatocytes (Fig. 5D). As in the duck hepatocytes described above (Fig. 2), infection with AdDHBV-L$^-$ led to hyperamplification of DHBV cccDNA in the *Tupaia* hepatocytes, suggesting a regulatory role of the DHBV L protein across the species barrier.

Our results clearly demonstrate that the adenovirus-mediated transfer of hepatitis B virus genomes easily and efficiently initiates HBV and DHBV replication across the species barrier, providing the means for studies on viral replication and its regulation in cells isolated from well-characterized experimental animals.

Adenovirus-mediated genome transfer establishes hepadnavirus replication in mice in vivo. To test whether an adenovirus-mediated genome transfer is suitable to initiate hepadnavirus replication in experimental animals in vivo, 8- to 12-week-old male C57BL/6 mice were injected in the tail vein with 2.5 × 10^9 efu of purified AdDHBV. At day 5 postinjection, mice were sacrificed and serum and liver samples were taken. Seven out of eight mice replicated DHBV, as shown by Southern blot analysis of total DNA extracted from the mouse livers (Fig. 6A). DHBV L (Fig. 6A) and core proteins (data not shown) were detected by Western blot analysis of liver lysates. All mice replicating DHBV were positive for DHBV DNA in the serum as shown by PCR analysis. The liver of the mouse not replicating DHBV was not successfully transduced by AdDHBV, as shown by PCR analysis of total liver DNA using adenovirus-specific primers (Fig. 6A). All mouse sera were negative for adenovirus DNA, excluding persistent circulation of AdDHBV.

The sera of these eight mice were shown to contain infectious DHBV particles by incubation of 10^6 primary duck hepatocytes with 50 μl of each serum and subsequent immunofluorescence staining as exemplified in Fig. 6B. No GFP expression was detected, again demonstrating the absence of circulating AdDHBV. In addition, infection was completely blocked by adding an anti-DHBV pre-S antiserum (44) (Fig. 6B) which blocked DHBV infection of primary duck hepatocytes in a 1:100 dilution but did not affect adenovirus infection

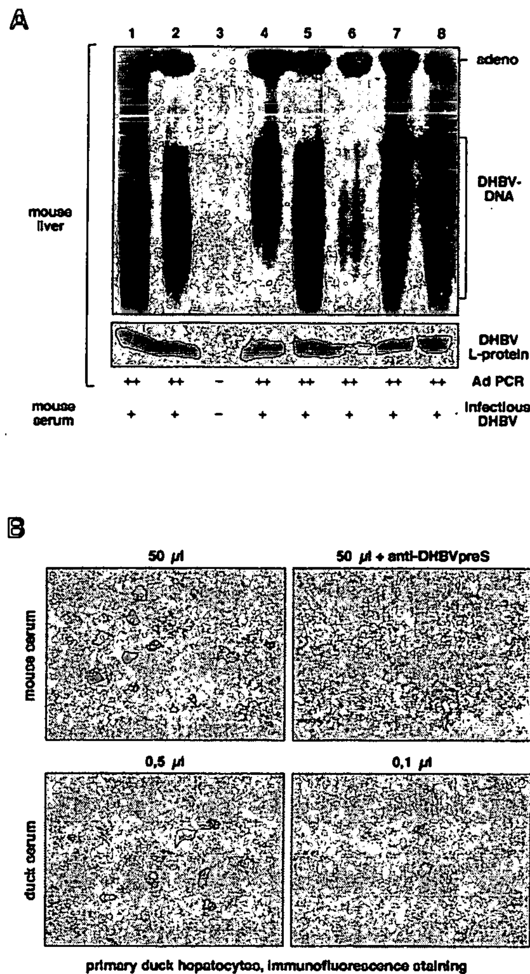

FIG. 6. In vivo infection of mice with AdDHBV. C57BL/6 mice were injected with 2.5×10^9 efu of AdDHBV (into the tail vein). (A) In seven out of eight mice, DHBV replicative DNA intermediates (DHBV DNA) were detected by Southern blot analysis of total liver DNA at day 5 p.i. High-molecular-weight bands correspond to the recombinant adenovirus genome (adeno). Intracellular DHBV L protein was detected by Western blot analysis of liver lysates. In the liver of the one mouse which did not replicate DHBV, no adenovirus DNA was detected by PCR analysis. All mice which replicated DHBV released infectious DHBV into their sera as tested by transfer onto primary duck hepatocytes. (B) To prove infectivity of the DHBV secreted into the mouse serum, primary duck hepatocytes were infected with 50 µl of mouse serum taken at day 5 post-AdDHBV infection (upper left panel) and analyzed by immunofluorescence staining for intracellular DHBV core protein. No GFP expression indicating contamination with AdDHBV was detected, and infectivity of the mouse serum was completely abolished by addition of a polyclonal anti-DHBV pre-S antiserum (+ anti-DHBVpreS; upper right panel). Infection efficiency was comparable to 0.5 µl of DHBV-positive duck serum containing 10^{10} DHBV-DNA equivalents/ml (lower panel). For better illustration, immunofluorescence stains are shown in an inverse fashion.

in control experiments (data not shown). By comparison to serum obtained from a DHBV-positive duck, DHBV DNA-containing particles detected in the mouse sera (10^8 DHBV genome equivalents per ml) were shown to be as infectious as those detected in duck serum (10^{10} DHBV genome equivalents per ml). Taken together, hepatitis B virus genomes were efficiently transferred to the livers of mice using adenovirus vectors and reproducibly induced virus replication in the experimental animal in vivo.

DISCUSSION

In this study, we demonstrate that adenovirus vectors allow the transduction of liver cells in cell culture and in livers of mice in vivo with replication-competent hepatitis B virus genomes, which efficiently initiate hepadnavirus replication and lead to secretion of infectious virions. We show that hepadnavirus cccDNA is established in addition to all other replicative intermediates following the adenovirus genome transfer. This indicates that HBV and DHBV replicate in transduced cells, at least in part independent from the transferred linear genome, using their natural transcription templates. The time course of viral replication and regulation of DHBV replication by the L protein were very similar to those observed in natural infection. The adenovirus-mediated genome transfer efficiently initiated HBV and DHBV replication across the species barrier and will allow studies of viral replication and its regulation in well-characterized experimental animals.

Furthermore, adenovirus-mediated transfer of hepatitis B virus genomes will allow us to study the ability of cells from various species to support HBV replication, to determine the replication competence of viral mutants, and to study the role of viral proteins in regulating the viral life cycle. The establishment of hepadnavirus cccDNA in heterologous cells following adenovirus genome transfer will enable us to study which step of the hepadnavirus replication cycle is supported by the respective cell and to analyze the cellular determinants. In the constructs used, HBV replication is initiated exclusively under control of the endogenous HBV promoters. This allows investigations of the replication competence of viral mutants and of the function of regulatory viral proteins by carefully directed knockouts.

Here we have provided evidence that in HBV, in contrast to DHBV (50), viral envelope proteins L and M are not responsible for regulation of nuclear reimport of progeny nucleocapsids, because HBV-L$^-$ genomes did not overamplify cccDNA, whereas DHBV-L$^-$ genomes did. These data are in accordance with observations in HBV-L$^-$ transgenic mice which contained <0.1 molecules of cccDNA per liver cell (C. Kuhn and H. Schaller, unpublished data). We further showed that HBV replication initiated in primary human hepatocytes by the adenovirus-mediated genome transfer was at least as efficient as that following infection with HBV virions (13, 16, 29, 41).

Methods to initiate hepatitis B virus replication include transfection of cloned hepadnavirus genomes into cultured cells (52), generation of stably transfected cells (47) or transgenic animals (18), baculovirus-mediated transfer of HBV genomes into hepatoma cells (8), and direct injection or cationic lipid-mediated transfer of naked viral DNA into the livers of animals (11, 12, 48, 53, 56). However, DNA transfection is not very efficient in most liver cell lines and inefficient in primary hepatocyte cultures. Unpredictable, high copy numbers of DNA molecules are introduced per cell.

Adenovirus-mediated genome transfer has distinct advantages. First, a broad range of immortalized and primary cells

can be transduced using adenovirus vectors and the amount of transgene can be controlled by varying the dose of recombinant adenovirus (42). Second, of all known gene delivery vectors, adenovirus vectors most efficiently transfer foreign DNA into the livers of a broad variety of experimental animals (4, 30). In the liver, they predominantly infect hepatocytes (22), the site of HBV replication in natural infection. Third, using adenovirus vectors, hepadnavirus replication is initiated from an extrachromosomal template as in natural infection.

Adenovirus genome transfer also compares favorably to recombinant baculoviruses, which have recently been reported as an alternative to transfer the 1.3-fold-overlength HBV genomes developed in our laboratory into cultured hepatoblastoma HepG2 cells (8). The level of HBV replication was adjusted by varying the amount of the recombinant virus used (8). Thus, a comparable amount of progeny HBV was released following infection with 20 efu of AdHBV in our study as with 200 PFU of HBV baculovirus per cell (9). In accordance with our observations, autonomous HBV replication was established following the transfer of a linearized genome.

There are, however, certain disadvantages of baculoviruses as vectors. Baculoviruses enter mammalian liver cells by an unspecific endosomal uptake rather than by receptor-mediated means (3, 23). In direct comparison to adenoviruses, baculoviruses transduce multiple DNA copies per cell (23). In addition, baculovirus-mediated gene transfer is restricted to certain species (23) and—most importantly—conventional baculovirus vectors are not suitable for gene transfer into experimental animals in vivo because they are rapidly inactivated by the complement system (24).

Adenovirus vectors, on the other hand, efficiently transduce liver cells in culture and in vivo (26). In this study we provide first evidence that adenovirus vectors are suitable for the establishment of small-animal models in which HBV replication is initiated from extrachromosomal hepatitis B virus genomes. The mouse probably is the most useful animal for the experimental analysis of various molecular and clinical aspects of HBV infection because mice are easy to breed and to keep, are genetically and immunologically well defined, and provide many available genetic variants. In AdHBV-infected mice, testing of viral mutants for their replication competence and relevance for pathogenesis in vivo will be easier and faster than with the alternative mouse models available so far (11, 18, 38, 40). Establishment of long-term virus replication and repeated applications, however, will probably be limited by a host immune response towards the adenovirus vectors (6, 26). In addition, the effects of adenovirus vectors on cell metabolism and cell growth and their cytotoxicity might restrict the application of the recombinant AdHBV to certain questions. To overcome the immune response, one will not necessarily have to work with immunosuppressed mice, which is technically demanding and allows studies on immunology and pathogenesis of hepatitis B virus infection only to a limited extent. To establish long-term HBV replication, one could take advantage of new developments in adenovirus vector technology (1), of short-term immunosuppression protocols (6) or of protocols specifically tolerizing the animals against the adenovirus vectors.

ACKNOWLEDGMENTS

The work was supported by a grant from the Deutsche Forschungsgemeinschaft (PR 618-1).

We thank Tong-Chuan He and Bert Vogelstein for providing the AdEasy system and Christa Kuhn for providing plasmid pHBV1.3L$^-$. We are grateful to Beate Zachmann-Brand for providing D1.3 constructs and to Bärbel Glass, Heiko Vogel, and Henning Schultze-Bergkamen for providing primary hepatocytes. We also thank Michael Kirschfink, Martina Müller-Schilling, Ernst Klar, and Peter Hofmann for excellent cooperation in obtaining primary human cells and Elizabeth Grgacic and Percy Knolle for helpful discussion.

REFERENCES

1. **Amalfitano, A.** 1999. Next-generation adenoviral vectors: new and improved. Gene Ther. **6**:1643–1645.
2. **Birnbaum, F., and M. Nassal.** 1990. Hepatitis B virus nucleocapsid assembly: primary structure requirements in the core protein. J. Virol. **64**:3319–3330.
3. **Boyce, F. M., and N. L. R. Bucher.** 1996. Baculovirus-mediated transfer into mammalian cells. Proc. Natl. Acad. Sci. USA **93**:2348–2352.
4. **Bramson, J. L., F. L. Graham, and J. Gauldie.** 1995. The use of adenoviral vectors for gene therapy and gene transfer in vivo. Curr. Opin. Biotechnol. **6**:590–595.
5. **Brown, J. J., B. Parashar, H. Moshage, K. E. Tanaka, D. Engelhardt, E. Rabbani, N. Roy-Chowdhury, and J. Roy-Chowdhury.** 2000. A long-term hepatitis B viremia model generated by transplanting nontumorigenic immortalized human hepatocytes in Rag-2-deficient mice. Hepatology **31**:173–181.
6. **Christ, M., M. Lusky, F. Stoeckel, D. Dreyer, A. Dieterle, A. I. Michou, A. Pavirani, and M. Mehtali.** 1997. Gene therapy with recombinant adenovirus vectors: evaluation of the host immune response. Immunol. Lett. **57**:19–25.
7. **Condreay, L. D., C. E. Aldrich, L. Coates, W. S. Mason, and T. T. Wu.** 1990. Efficient duck hepatitis B virus production by an avian liver tumor cell line. J. Virol. **64**:3249–3258.
8. **Delaney, W. E., IV, and H. C. Isom.** 1998. Hepatitis B virus replication in human HepG2 cells mediated by hepatitis B virus recombinant baculovirus. Hepatology **28**:1134–1146.
9. **Delaney, W. E., IV, T. G. Miller, and H. C. Isom.** 1999. Use of the hepatitis B virus recombinant baculovirus-HepG2 system to study the effects of (−)-β-2′,3′-dideoxy-3′-thiacytidine on replication of hepatitis B virus and accumulation of covalently closed circular DNA. Antimicrob. Agents Chemother. **43**:2017–2026.
10. **Di, Q., J. Summers, J. B. Burch, and W. S. Mason.** 1997. Major differences between WHV and HBV in the regulation of transcription. Virology **229**: 25–35.
11. **Eto, T., and H. Takahashi.** 1999. Enhanced inhibition of hepatitis B virus production by asialoglycoprotein receptor-directed interferon. Nat. Med. **5**:577–581.
12. **Feitelson, A. M., L. J. DeTolla, and X. D. Zhou.** 1988. A chronic carrierlike state is established in nude mice injected with cloned hepatitis B virus DNA. J. Virol. **62**:1408–1415.
13. **Galle, P. R., J. Hagelstein, B. Kommerell, M. Volkmann, P. Schranz, and H. Zentgraf.** 1994. In vitro experimental infection of primary human hepatocytes with hepatitis B virus. Gastroenterology **106**:664–673.
14. **Galle, P. R., H. J. Schlicht, C. Kuhn, and H. Schaller.** 1989. Replication of duck hepatitis B virus in primary duck hepatocytes and its dependence on the state of differentiation of the host cell. Hepatology **10**:459–465.
15. **Ganem, D.** 1996. *Hepadnaviridae*: the viruses and their replication, p. 2703–2737. *In* B. N. Fields, D. M. Knipe, and P. M. Howley (ed.), Fields virology, 3rd ed., vol. 2. Lippincott-Raven Publishers, Philadelphia, Pa.
16. **Gripon, P., C. Diot, and C. Guguen-Guillouzo.** 1993. Reproducible high level infection of cultured adult human hepatocytes by hepatitis B virus: effect of polyethylene glycol on adsorption and penetration. Virology **192**:534–540.
17. **Guidotti, L. G., P. Borrow, M. V. Hobbs, B. Matzke, I. Gresser, M. B. Oldstone, and F. V. Chisari.** 1996. Viral cross talk: intracellular inactivation of the hepatitis B virus during an unrelated viral infection of the liver. Proc. Natl. Acad. Sci. USA **93**:4589–4594.
18. **Guidotti, L. G., B. Matzke, H. Schaller, and F. V. Chisari.** 1995. High-level hepatitis B virus replication in transgenic mice. J. Virol. **69**:6158–6169.
19. **Guidotti, L. G., R. Rochford, J. Chung, M. Shapiro, R. Purcell, and F. V. Chisari.** 1999. Viral clearance without destruction of infected cells during acute HBV infection. Science **284**:825–829.
20. **He, T. C., S. Zhou, L. T. da Costa, J. Yu, K. W. Kinzler, and B. Vogelstein.** 1998. A simplified system for generating recombinant adenoviruses. Proc. Natl. Acad. Sci. USA **95**:2509–2514.
21. **Heermann, K. H., U. Goldmann, W. Schwartz, T. Seyffarth, H. Baumgarten, and W. H. Gerlich.** 1984. Large surface proteins of hepatitis B virus containing the pre-*s* sequence. J. Virol. **52**:396–402.
22. **Hegenbarth, S., R. Gerolami, U. Protzer, P. L. Tran, C. Brechot, G. Gerken, and P. A. Knolle.** 2000. Liver sinusoidal endothelial cells are not permissive for adenovirus type 5. Hum. Gene Ther. **11**:481–486.

23. **Hofmann, C., V. Sandig, G. Jennings, M. Rudolph, P. Schlag, and M. Strauss.** 1995. Efficient gene transfer into human hepatocytes by baculovirus vectors. Proc. Natl. Acad. Sci. USA **92:**10099–10103.
24. **Hofmann, C., and M. Strauss.** 1998. Baculovirus-mediated gene transfer in the presence of human serum or blood facilitated by inhibition of the complement system. Gene Ther. **5:**531–536.
25. **Hoofnagle, J. H., and A. di Bisceglie.** 1997. The treatment of chronic viral hepatitis. N. Engl. J. Med. **336:**347–356.
26. **Ilan, Y., H. Saito, N. R. Thummala, and N. R. Chowdhury.** 1999. Adenovirus-mediated gene therapy of liver diseases. Semin. Liver Dis. **19:**49–59.
27. **Kay, A. M., D. Liu, and P. M. Hoogerbrugge.** 1997. Gene therapy. Proc. Natl. Acad. Sci. USA **94:**12744–12746.
28. **Lau, D. T., J. Everhart, D. E. Kleiner, Y. Park, J. Vergalla, P. Schmid, and J. H. Hoofnagle.** 1997. Long-term follow-up of patients with chronic hepatitis B treated with interferon alfa. Gastroenterology **113:**1660–1667.
29. **Le Seyec, J., P. Chouteau, I. Cannie, C. Guguen-Guillouzo, and P. Gripon.** 1998. Role of the pre-S2 domain of the large envelope protein in hepatitis B virus assembly and infectivity. J. Virol. **72:**5573–5578.
30. **Li, Q., M. A. Kay, M. Finegold, L. D. Stratford-Perricaudet, and S. L. Woo.** 1993. Assessment of recombinant adenoviral vectors for hepatic gene therapy. Hum. Gene Ther. **4:**403–409.
31. **Liu, H. S., M. S. Jan, C. K. Chou, P. H. Chen, and N. J. Ke.** 1999. Is green fluorescent protein toxic to the living cells? Biochem. Biophys. Res. Commun. **260:**712–717.
32. **Loser, P., V. Sandig, I. Kirillova, and M. Strauss.** 1996. Evaluation of HBV promoters for use in hepatic gene therapy. Biol. Chem. Hoppe-Seyler **377:**187–193.
33. **Mabit, H., and H. Schaller.** 2000. Intracellular hepadnaviral nucleocapsids are selected for secretion by envelope protein-independent membrane binding. J. Virol. **74:**11472–11478.
34. **Mandart, E., A. Kay, and F. Galibert.** 1984. Nucleotide sequence of a cloned duck hepatitis B virus genome: comparison with woodchuck and human hepatitis B virus sequences. J. Virol. **49:**782–792.
35. **Nassal, M., and H. Schaller.** 1996. Hepatitis B virus replication—an update. J. Viral Hepat. **3:**217–226.
36. **Niederau, C., T. Heintges, S. Lange, G. Goldmann, C. Niederau, L. Mohr, and D. Haussinger.** 1996. Long-term follow-up of HBeAg-positive patients treated with interferon alpha for chronic hepatitis B. N. Engl. J. Med. **334:**1422–1427.
37. **Obert, S., B. Zachmann-Brandt, E. Deindl, W. Tucker, R. Bartenschlager, and H. Schaller.** 1996. A spliced hepadnaviral RNA that is essential for virus replication. EMBO J. **15:**2565–2574.
38. **Ohashi, K., P. L. Marion, H. Nakai, L. Meuse, J. M. Cullen, B. B. Bordier, R. Schwall, H. B. Greenberg, J. S. Glenn, and M. A. Kay.** 2000. Sustained survival of human hepatocytes in mice: a model for in vivo infection with human hepatitis B and hepatitis delta viruses. Nat. Med. **6:**327–331.
39. **Pasek, M., T. Goto, W. Gilbert, B. Zink, H. Schaller, P. MacKay, G. Leadbetter, and K. Murray.** 1979. Hepatitis B virus genes and their expression in E. coli. Nature **282:**575–579.
40. **Petersen, J., M. Dandri, S. Gupta, and C. E. Rogler.** 1998. Liver repopulation with xenogenic hepatocytes in B and T cell-deficient mice leads to chronic hepadnavirus infection and clonal growth of hepatocellular carcinoma. Proc. Natl. Acad. Sci. USA **95:**310–315.
41. **Protzer, U., M. Nassal, P. W. Chiang, M. Kirschfink, and H. Schaller.** 1999. Interferon gene transfer by a hepatitis B virus vector efficiently suppresses wild-type virus infection. Proc. Natl. Acad. Sci. USA **96:**10818–10823.
42. **Ragot, T., P. Opolon, and M. Perricaudet.** 1997. Adenoviral gene delivery. Methods Cell Biol. **52:**229–260.
43. **Roggendorf, M., and T. K. Tolle.** 1995. The woodchuck: an animal model for hepatitis B virus infection in man. Intervirology **38:**100–112.
44. **Schlicht, H. J., C. Kuhn, B. Guhr, R. J. Mattaliano, and H. Schaller.** 1987. Biochemical and immunological characterization of the duck hepatitis B virus envelope proteins. J. Virol. **61:**2280–2285.
45. **Schlicht, H. J., J. Salfeld, and H. Schaller.** 1987. The duck hepatitis B virus pre-C region encodes a signal sequence which is essential for synthesis and secretion of processed core proteins but not for virus formation. J. Virol. **61:**3701–3709.
46. **Schödel, F., T. Weimer, D. Fernholz, R. Schneider, R. Sprengel, G. Wildner, and H. Will.** 1991. The biology of avian hepatitis B viruses, p. 53–80. *In* A. McLachlan (ed.), Molecular biology of the hepatitis B virus. CRC Press, Boca Raton, Fla.
47. **Sells, A. M., M. L. Chen, and G. Acs.** 1987. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. Proc. Natl. Acad. Sci. USA **84:**1005–1009.
48. **Sprengel, R., C. Kuhn, C. Manso, and H. Will.** 1984. Cloned duck hepatitis B virus DNA is infectious in Peking ducks. J. Virol. **52:**932–937.
49. **Summers, J., and W. S. Mason.** 1982. Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell **29:**403–415.
50. **Summers, J., P. M. Smith, and A. L. Horwich.** 1990. Hepadnavirus envelope proteins regulate covalently closed circular DNA amplification. J. Virol. **64:**2819–2824.
51. **Summers, J., P. M. Smith, M. J. Huang, and M. S. Yu.** 1991. Morphogenetic and regulatory effects of mutations in the envelope proteins of an avian hepadnavirus. J. Virol. **65:**1310–1317.
52. **Sureau, C., J. L. Romet-Lemonne, J. I. Mullins, and M. Essex.** 1986. Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. Cell **47:**37–47.
53. **Takahashi, H., J. Fujimoto, S. Hanada, and K. J. Isselbacher.** 1995. Acute hepatitis in rats expressing human hepatitis B virus transgenes. Proc. Natl. Acad. Sci. USA **92:**1470–1474.
54. **Tuttleman, J. S., J. C. Pugh, and J. W. Summers.** 1986. In vitro experimental infection of primary duck hepatocyte cultures with duck hepatitis B virus. J. Virol. **58:**17–25.
55. **Walter, E., R. Keist, B. Niederost, I. Pult, and H. E. Blum.** 1996. Hepatitis B virus infection of Tupaia hepatocytes in vitro and in vivo. Hepatology **24:**1–5.
56. **Will, H., R. Cattaneo, G. Darai, F. Deinhardt, H. Schellekens, and H. Schaller.** 1985. Infectious hepatitis B virus from cloned DNA of known nucleotide sequence. Proc. Natl. Acad. Sci. USA **82:**891–895.
57. **World Health Organization.** 1996. Viral Hepatitis Prevention Board: prevention and control of hepatitis B in the community. W. H. O. Communicable Ser. **1:**1–26.
58. **Yan, R. Q., J. J. Su, D. R. Huang, Y. C. Gan, C. Yang, and G. H. Huang.** 1996. Human hepatitis B virus and hepatocellular carcinoma. I. Experimental infection of tree shrews with hepatitis B virus. J. Cancer Res. Clin. Oncol. **122:**283–288.
59. **Zoulim, F.** 1999. Therapy of chronic hepatitis B virus infection: inhibition of the viral polymerase and other antiviral strategies. Antivir. Res. **44:**1–30.
60. **Zuckerman, A. J., and D. Lavanchy.** 1999. Treatment options for chronic hepatitis. Antivirals look promising. BMJ **319:**799–800.